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An inducible transposon system to terminate the function of a selectable marker in transgenic plants

Yuh-Chyang Charng · Kuan-Te Li · Hung-Kuang Tai · Na-Sheng Lin · Jenn Tu

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Abstract Since the maize transposon *Ac* can move to a new location within the genome, it has been used in removing selectable markers in transgenic plants. In this paper, we developed an inducible transposon system to truncate a selectable marker in transgenic plants. In this system, the marker gene was accompanied by the inducible transposon, but one end of the transposon was located in the intron of the marker gene. As an example of a marker gene, we isolated the rice 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) and modified it for glyphosate tolerance. The transposon contained *Ac* transposase, which fused with the promoter of the inducible gene for pathogenesis-related protein 1a (PR-1a). This construct was engineered into an expression vector pCAMBIA1300, harboring a hygromycin-resistant gene. The construct was first transformed into rice calli, and transformed plants were selected on hygromycin-containing medium. The stably transformed calli underwent determination for normal transcripts and tolerance to glyphosate. The results were applied to a rice transformation with the same construct, but using glyphosate as the selective agent. By determining the transformation efficiency, T-DNA copy patterns, we demonstrate that the modified *epsps* could be a suitable selectable marker to create transgenic rice. Furthermore, after obtaining stable transgenic plants and inducing transposition by salicylic acid, the transposon was excised, the marker gene became truncated, and its expression was terminated. This strategy could be applicable to yield self-stabilizing transposon by locating the transposon's end in the transposase gene's intron.

Keywords *Ac* transposase · *epsps* · Inducible transposon · Selectable marker · Transgenic plants

Abbreviations

epsps	5-enolpyruvylshikimate-3-phosphate synthase gene
GOI	Gene of interest
SA	Salicylic acid
TPase	Transposase gene

Introduction

The genetic modification of plants offers improvements in agricultural practices, food safety, and human health. The development of transgenic plants

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Y.-C. Charng (✉) · K.-T. Li · H.-K. Tai
Department of Agronomy, National Taiwan University, No. 1 Sec. 4 Roosevelt Rd., Taipei, Taiwan, Republic of China
e-mail: bocharng@ntu.edu.tw

N.-S. Lin · J. Tu
Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, Republic of China

requires the use of selectable marker genes, because the efficiency of plant transformation is less than optimal for many important plant species (Hohn et al. 2001). In current plant transformation systems, a selectable marker gene is co-delivered with the gene of interest (GOI) to identify and separate rare transgenic cells from non-transgenic cells.

Usually, a conditional dominant gene, with no influence on the growth or morphology of plants, is used as a selectable marker (Endo et al. 2002). Dominant genes encoding either antibiotic or herbicide resistance are widely used as selectable markers. The antibiotics and herbicides used to select rare transgenic cells from non-transgenic cells generally have negative effects on proliferation and differentiation (Ebinuma et al. 1997). For example, glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; E. C. 2.5.1.19), a critical enzyme in the shikimate pathway for the biosynthesis of aromatic amino acids (Bradshaw et al. 1997; Schönbrunn et al. 2001). Glyphosate-resistant *epsps* was successfully used as a selectable marker in the plant transformation of oilseed rape, soybean, potato, maize, and wheat (Clemente et al. 2000; Hu et al. 2003; Miki and McHugh 2004; Zhou et al. 1995).

Over the past few years, consumer and environmental groups have expressed concern about the use of markers from an ecological and food safety perspective. The development of marker-free transgenic plants is desirable in agricultural biotechnology. Many strategies to produce marker-free transgenic plants have been described (reviewed by Ebinuma et al. 2001; Hare and Chua 2002; Hohn et al. 2001; Miki and McHugh 2004). The Cre-*lox* site-specific recombination system has been widely studied for marker removal. In fact, the first marker-free commercial transgenic plant was developed using Cre-*lox* technology (Ow 2007). In the transformation vector, the marker is flanked by directly oriented *lox* sites. A *cre* gene is introduced into the genome from a genetic cross. The expression of the Cre protein causes recombination between the two *lox*P sites, and the marker is lost during the process of recombination. New techniques have been developed to control the *cre* gene expression by flanked with an inducible promoter (Zuo et al. 2001). A limitation of this system is that the high level of expression of the *cre* gene may result in phenotypic aberrations in some plant species (Hajdukiewicz et al. 2001).

In contrast to the Cre-*lox* recombination system leading to the loss of the marker gene, the transposon system (e.g., *Ac*/*Ds*) offers information about the new location of the removed marker's DNA. The maize transposon *Ac* is an autonomous transposable element of 4565 bp and is active in a wide range of plant species. It codes for a single gene product, *Ac* transposase, which, together with the inverted repeats and about 250 bp of both ends (terminal regions) of the transposon and putative host factors, is the only prerequisite for transposition of the *Ac* element in plants (Haring et al. 1991). In the transformation vector, the marker gene is inserted into the *Ds* element. The expression of the *Ac* transposase excises both ends of the transposon and usually re-integrates into other locations on the chromosome. When the transposon transposes within the same chromosome (linked transposition), both insertion sites of the T-DNA (harboring the marker gene) and the transposon (harboring the GOI) need regulatory approval for commercialization. With unlinked transposition, the marker gene can be removed by out-crossing. Although the work is time consuming, all removed information remains clear for regulatory approval. Furthermore, with the transposon system, one successful transformation can create more independent transgenic lines because of the re-integrated loci. This feature is valuable for creating transgenic plants in species with low transformation efficiency. However, out-crossing with this system cannot be used with vegetatively propagated plants and woody tree species. According to this, we designed a system to truncate a marker gene after transposition, termed "marker-off", which respects the marker-free system and does not require segregating the marker away. The system involves introducing an intron-containing marker gene, accompanied by a transposon whose one end is located in the intron of the marker gene, into a transformation vector. The prerequisite of the strategy is to obtain an intron-containing marker gene. Since Howe et al. (2002) successfully modified the maize *epsps* as a selectable marker for creating transgenic maize, we expected that a modified rice (*Oryza sativa* L.) *epsps* could serve as a selectable marker for the transformation of rice. For the transposon to terminate the marker, we applied a chemical inducible transposon. Previously, the *Ac* transposase gene was fused with the promoter of the pathogenesis-related protein 1a (PR-1a). PR-1

proteins are induced in plants as a consequence of the hypersensitive defense reaction elicited by pathogen infection (van Loon and van Kammen 1970), by exogenous application of some chemicals, e.g., salicylic acid (SA) (White 1979), and by developmental stimuli (Gruener and Pfitzner 1994). This PR-1a-transposase fusion was inserted in a *Ds* element to yield an inducible transposon (Charng et al. 2000). Here, the 5′ end of this inducible transposon was constructed in the first intron of *epsps*. We studied the expression pattern of the modified *epsps* and glyphosate resistance activity in transgenic rice, and then determined the rice transformation efficiency and T-DNA integration copies when glyphosate was used as the selection agent. Finally, we induced transposition events to terminate the glyphosate-tolerance ability in transgenic rice.

Materials and methods

Modification of the rice *epsps*

Plasmid p6140 containing a genomic copy of *epsps* from rice was a gift from Dr. Kishima (Hokkaido University, Japan). To construct a gene for the glyphosate-tolerant form of the EPSPS enzyme, we used site-directed mutagenesis to change two codons in the coding sequence as described previously (Howe et al. 2002), whereby the authors modified the corn *epsps* cDNA clone for a glyphosate-tolerant form. The DNA fragment containing exons 2–4 was firstly sub-cloned into plasmid pBC. The codon for the glycine residue at position 168 was changed to encode an alanine residue and that for the glycine residue at position 211 was changed to encode an aspartic acid residue by polymerase chain reaction (PCR) as described. The resulting DNA fragment, together with the other *epsps* exons, was used for subsequent construction.

DNA manipulation and plant transformation

The construction procedures for the KCEH system (*KCEH* transposon plus the marker gene) is depicted in the supplementary material. All cloning and DNA manipulations followed standard procedures (Sambrook and Russel 2001) with the use of chemicals from Roche (Basel, Switzerland). All transformations involved the use of rice (*Oryza sativa* L. cv. TNG67) as described (Toki 1997).

Assay of T1 progeny resistant to glyphosate

Successful transgenic plants resistant to glyphosate were self-pollinated to obtain T1 seeds. The seeds were imbibed in flowing water for 2 days, then transferred to an iron grid and kept in water for 2 weeks, and then soil for another week. The three leaf-age stage rice seedlings were sprayed once with 5,000 ppm Roundup®. The effect could be observed after 1 week.

RT-PCR analysis of *epsps* expression

RNA extracted from the transgenic rice calli was reverse transcribed with use of the SuperScript™ First-Strand Synthesis System (Invitrogen). Primers specific to each of the two target messages were used in subsequent PCR amplification: R-E1FC (5′-ATCGTGCTCCAG CCCATCAG-3′), mKRT1F (5′-CAACTCTTCTTGG GGAACGCTGCT-3′), and mKRT2R (5′-CAAGGAA ACAGTCGACATCCGCGT-3′). Primers specific to endogenous *epsps* were KRT1F (5′-CAACTCTTCT TGGGGAACGCTGGA-3′) and KRT2R (5′-CA AGGAAACAGTCGACATCCGCAC-3′).

Induction of *KCEH* transposition and the determination of insertion sites

For induction experiments, T1 rice seeds of each transformed line harboring a single copy of *KCEH* were incubated on callus induction medium (CIM) containing hygromycin for 4 weeks in order to yield enough calli for the induction experiments. We previously found 5 mM of SA to have the highest induction efficiency in transgenic rice containing a PR-1a-based transposon (Charng et al. 2007), so we induced transposition by incubating transgenic rice calli harboring the KCEH system on CIM containing 5 mM of SA. The empty donor sites of transposition were determined with the primers CAMBIA1 (5′-GTCGACTTTCTAGAGGATCCG-3′) and mKRT2R (5′-CAAGGAAACAGTCGACATCCGCGT-3′).

The flanking sequences of the *KCEH* element in transgenic plants were amplified by TAIL-PCR (Liu et al. 1995; Sha et al. 2004) with the following oligonucleotide primers: 3–1 (5′-GTGTGCTCCAGATT TATATGG-3′), 3–2 (5′-GATTTCGACTTTAACCCG ACCGGA-3′), and 3–3 (5′-CGTTTTCGTTACCGG TATATCCCG-3′) for the 3′ end. The arbitrary degenerate (AD) primers and TAIL-PCR were as described previously (Liu et al. 1995; Sha et al. 2004), with following modification: the primary TAIL-PCR contained approximately 150 ng of rice genomic DNA.

Results

Construction of the transposon-mediated marker-off system

The construction of the inducible transposon system to truncate the marker gene is depicted in Fig. 1a. First, the 5′ end of the *Ac* element was inserted in the first intron of the rice genomic *epsps* driven by the CaMV35S promoter, which had been modified to produce a glyphosate-tolerant form of the enzyme. To complete a new transposon, a PR-1a::*TPase* fusion was flanked by the 3′ end of *Ac*. The new transposon, *KCEH*, contains a PR-1a::*TPase* fusion, a CaMV35S promoter, and the first exon of the modified *epsps*. The KCEH system (*KCEH* transposon plus the marker gene) was inserted into the binary vector pCAMBIA 1300, yielding the plasmid pKCEH, which was introduced into the *Agrobacterium tumefaciens* strain LBA4404 for plant transformation.

Expression of the modified *epsps* in transgenic rice plants

To determine the behavior of the modified *epsps* in rice, the KCEH construct was transformed into rice, with hygromycin used as the selection agent. A total of 21 single-copy transgenic lines were determined by PCR and DNA blot analysis (data not shown), and the total RNA of each line was extracted for RT-PCR. The modified *epsps* in the KCEH construct and endogenous rice *epsps* differ in 4 bp. To eliminate the possibility of amplifying the endogenous rice *epsps*, we designed two primers, mKRT1F and mKRT2R,

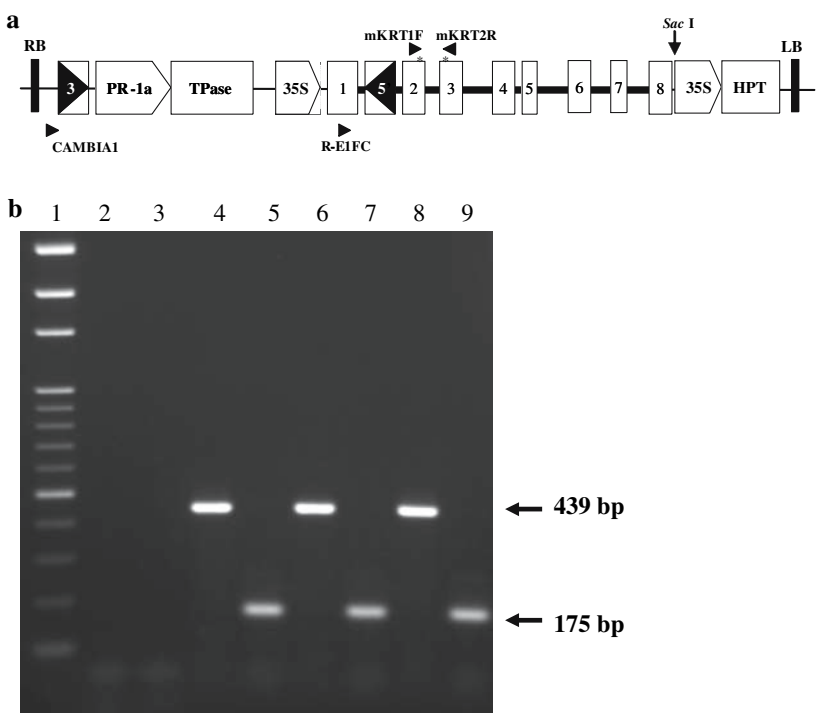

Fig. 1 (**a**) Schematic representation of the KCEH system and the location of primers (shown as *solid triangles*). LB=left border; RB = right border; 5′ and 3′ = *Ac* left and right terminal-inverted repeat; PR-1a = inducible promoter; *TPase* = transposase gene; 35S = cauliflower mosaic virus 35S RNA promoter; 1 ~ 8 = *epsps* exon 1 ~ exon 8; bold lines = *epsps* introns; *mutation site. (**b**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of modified (or endogenous) *epsps* expression in transgenic rice (lanes 4, 5, 8, 9) or wild type (lanes 2, 3, 6, 7) with primers R-E1FC and mKRT2R (lanes 2 and 4), mKRT1F and mKRT2R (lanes 3 and 5), R-E1FC and KRT2R (lanes 6 and 8), KRT1F and KRT2R (lanes 7 and 9). Lane 1: 100-bp marker

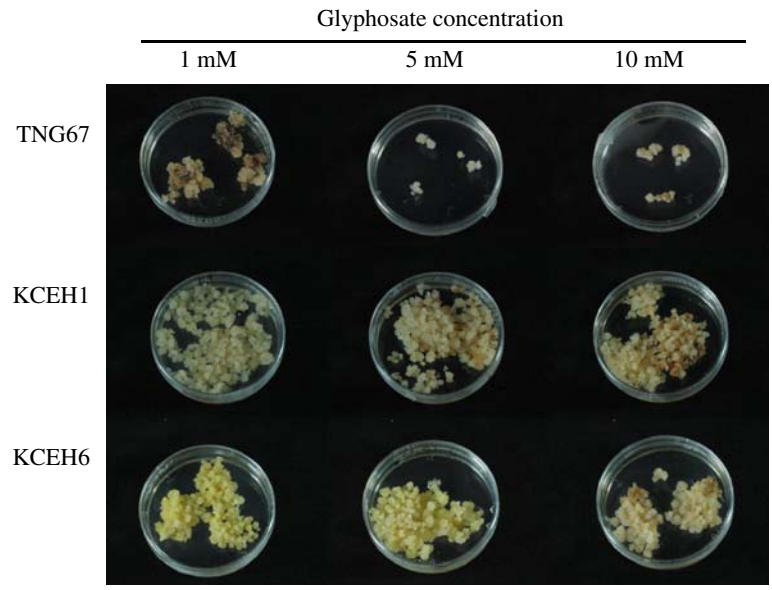

Fig. 2 Determination of the glyphosate concentration of the modified *epsps* as a selective marker. Each line was cultured in callus-induced medium under different concentrations of glyphosate (1, 5, and 10 mM) for 5 weeks. KCEH-H1 and KCEH-H6 are independent transgenic lines with hygromycin used as the selection agent. TNG67, wild-type rice

which were specific to the modified sites of *epsps*. These two primers are mismatched at the two 3′ terminal bases for endogenous *epsps* and have more power to identify transgenes. To ensure functional splicing of the first intron by PCR, the primers R-E1FC and mKRT2R were designed from the first and third exons of the modified *epsps*. In normal splicing of the endogenous *epsps*, the predictive fragment is 439 bp (Fig. 1b). With mKRT1F and mKRT2R, only modified *epsps* could be amplified by PCR, and the predicted fragment 175 bp was amplified (Fig. 1b). The experiments were performed again but primers mKRT1F and mKRT2R were replaced with primers KRT1F and KRT2R, which are matched for endogenous *epsps*. With these two primers, the expected DNA fragments could be amplified in transgenic rice, as well as in wild type TNG67.

Efficient *Agrobacterium*-mediated transformation of rice with modified *epsps*

To determine whether the modified *epsps* can be used as a selectable marker in rice, the regenerated calli of transgenic lines containing a single copy of the modified *epsps* and the wild type were incubated with CIM containing glyphosate (1, 5, or 10 mM) for 4 weeks. Figure 2 shows the tolerance of transgenic calli lines up to 10 mM of glyphosate, with the growth of wild-type calli extremely restricted on media containing 5 mM or 10 mM of glyphosate. Thus, we used 5 mM of glyphosate as the selection condition for rice transformation.

The pKCEH construct was introduced into the embryonic calli of rice, and transgenic plants were screened with use of 5 mM of glyphosate. The transgenic calli differed from nontransgenic calli by their proliferation potential under glyphosate treatment (Fig. 3a). The transgenic calli were transferred to differentiation medium to set shoots (Fig. 3b), then, the resulting plantlets were transferred to the soil and grown in the greenhouse to set seeds (Fig. 3c). We obtained 98 transgenic lines derived from 1,086 calli pools, for 9.02% transformation efficiency. To verify stable transformation, *Sac* I-digested genomic DNA from each independent transgenic line underwent Southern blot analysis. Since the modified *epsps* differs from the endogenous *epsps* by only 4 bp, we used the 1.1-kb fragment of the *hpt* gene as a probe. As an example, in Fig. 4, 9 of 20 transgenic lines contained only one T-DNA copy and the others more than two copies. From 98 transgenic lines, 43 single-copy independent transgenic rice lines were obtained, which indicates that the selection procedure is suitable for creating single-copy lines. To determine the inheritance of the modified *epsps* and whether the transgenic rice plants were resistant to Roundup® (glyphosate applied in the field), mature rice seedlings from the T1 progeny were sprayed with 5,000 ppm Roundup®. We

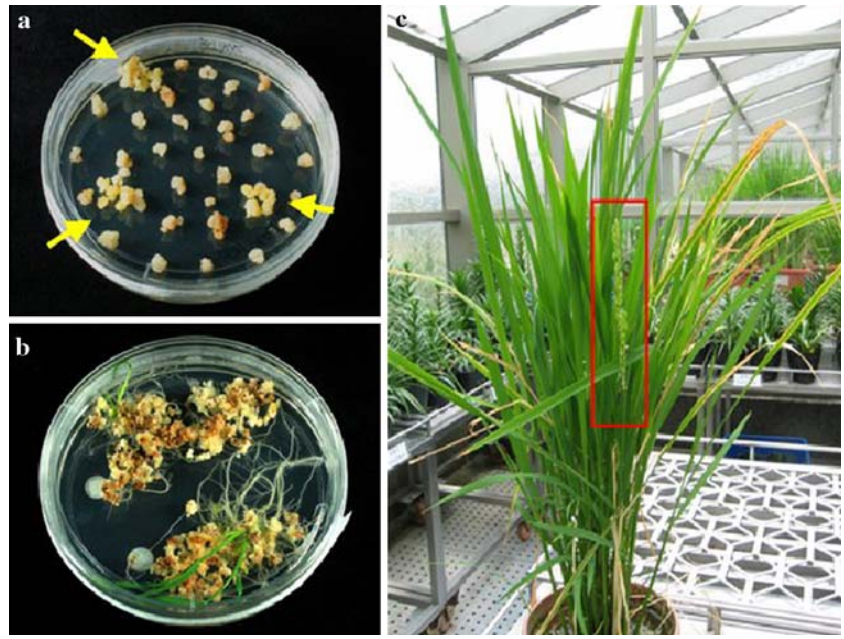

Fig. 3 Different periods in the modified rice *epsps* transformation system. (**a**) Proliferation of calli in 5-mM of glyphosate growth medium over 5 weeks (*yellow arrows*). (**b**) Differentiation of proliferating calli into shoots and roots in shooting medium over 5 weeks. (**c**) Transformants were transferred to soil for 2 months, grew well, and headed normally (*red box*)

observed the expected 3:1 Mendelian ratio for the modified *epsps* among all single-copy T-DNA transgenic lines (data not shown; another progeny assay result is described below). Thus, the glyphosate-tolerant phenotype was inherited as a single Mendelian locus in all of the plants tested. All of these results indicate that the modified *epsps* is suitable as a selectable marker in rice.

Transposition of *KCEH* and termination of the glyphosate-tolerant *epsps* in transgenic rice

Previously, we introduced an *Ac*-based inducible transposon, *INAc*, into rice and found that the highest transposition efficiency was induced with 5 mM of SA (Charng et al. 2007). Thus, to remove the functional glyphosate-tolerant *epsps*, we applied 5 mM of SA to transgenic rice calli to trigger the *KCEH* transposon. Calli regenerated from the T1 rice seeds of each transformed line harboring a single copy of *KCEH* were incubated on CIM containing 5 mM of SA to induce transposition. The excision events were determined by PCR with the primers mKRT2R and CAMBIA1. To determine the empty donor site, a 550-bp DNA fragment was expected (Fig. 5a). Of 43 single-copy lines, seven transgenic lines yielded the expected 550-bp DNA products, for 16% somatic transposition efficiency. Sequencing analysis confirmed the residual DNA after the excision of the transposon (Fig. 5b).

According to our previous studies of transposition events in rice, *Ac*-based inducible transposons are very active in induced rice calli, but, sometimes, only a portion of cells contain the empty donor site (partial transposition events; Charng et al. 2007). We determined whether the transposition events passed through the germ line and were inherited in the progeny (germinal transposition) or not (somatic transposition). The remaining calli of the transposed lines, as well as non-SA-treated calli (controls), were cultured to set shoots and then transplanted to soil for self-pollination. The seedlings of the progeny underwent PCR to determine the inheritance of the transposition events. Of seven lines showing transposition, only two showed the transposition events inherited in the progeny. The seedlings of the progeny were treated with Roundup® as described above. All 25 seedlings were glyphosate-sensitive, which indicates the loss of the glyphosate-tolerant function (Fig. 6 left). As a control, siblings of the same transgenic line which had not been induced with SA for transposition were cultured to harvest self-pollinated seeds for glyphosate-tolerant assay and showed glyphosate-tolerance as a single Mendelian locus pattern (Fig. 6 right).

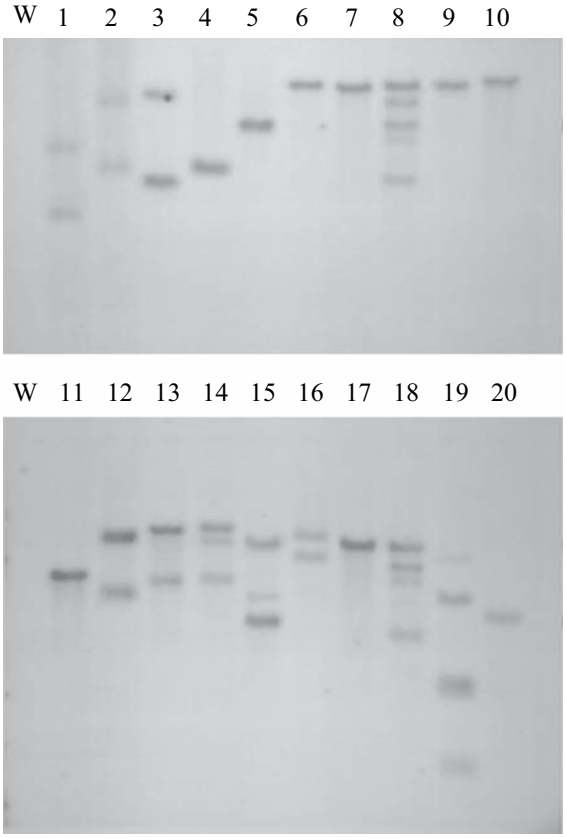

Fig. 4 Analysis of T-DNA copies of transgenic rice selected by 5 mM of glyphosate. Instead of the use of modified *epsps*, *hpt* was used as a probe for DNA blot analysis because of the presence of endogenous *epsps*. W=wild type TNG67 rice; 1–20 represent the selected plants from T0 glyphosate-tolerant transformants. The single-copy T-DNA transgenic lines were re-numbered for subsequent analysis

Genomic DNA containing the independent transposed *KCEH* was collected to amplify the flanking sequences of the transposed *KCEH* elements. The flanking sequences were isolated by TAIL PCR. A summary of the significant homologies obtained after comparison of the flanking sequences from a public database is shown in Table 1. Of six independent transposition events, four showed linkages and one no linkage to the T-DNA locations. For one event, the flanking sequence was not obtained, possibly because of failure to amplify the PCR product or loss of the transposon after excision from its donor site. Taken together, the results indicate that the KCEH system offers a desirable selectable marker for rice transformation and the ability to remove the marker thereafter.

Discussion

Marker-free systems investigated in transgenic plants include the use of co-transformed genes, site-specific recombination, and transposon mediation. Co-transformed and transposon-mediated systems enable the GOI and marker to integrate into different loci in the plant genome. Unlinked marker genes can then be segregated away from the GOI to produce marker-free transgenic plants (reviewed by Ebinuma et al. 2001), but this technology is not useful for woody plants or plants that reproduce vegetatively. Site-specific recombination systems remove the marker gene by the use of single enzymes (e.g., Cre) acting on specific target sequences. Each of the target sites contain a few oligonucleotides surrounded by a few inverted repeats to determine the orientation of the target site. The expression of the recombinase causes recombination between the target sites and results in the loss of the marker gene flanked by the sites. However, high levels of recombinase expression may result in genome rearrangements at cryptic-target sites. Although such sites have not been described in the nuclear genomes of plants, chloroplast cryptic *lox* sites have been described (Hajdukiewicz et al. 2001). In contrast to the short target recognition sequences (e.g., 34 bp for *lox*P sites), the existence of pseudo-target sites for transposons (e.g., about 250 bp for *Ac*/*Ds*) is believed to be rare in the plant genome. We, therefore, applied an inducible transposon technology to develop a marker-off system without the need for out-crossing. The new system is called KCEH and contains the *Ac*-based inducible transposon *KCEH* and the marker gene, modified *epsps*. We inserted one end of *KCEH* into the first intron of the marker and then introduced it into the rice genome; *epsps* was expressed functionally (Figs. 1, 2, and 6). Yet, when the transposon was excised after induced transposition, the DNA sequences within both ends were removed and the marker was truncated and lost its function.

To study the KCEH system, we first determined whether the modified *epsps* was a useful marker. Although the first intron was inserted by the 5′ end of the transposon, Fig. 1b and the sequence analysis (data not shown) indicated that the splice junction of the first intron could be identical to the junction of the native *epsps*. Figure 2 demonstrated the glyphosate-tolerance function of the modified *epsps* in transgenic

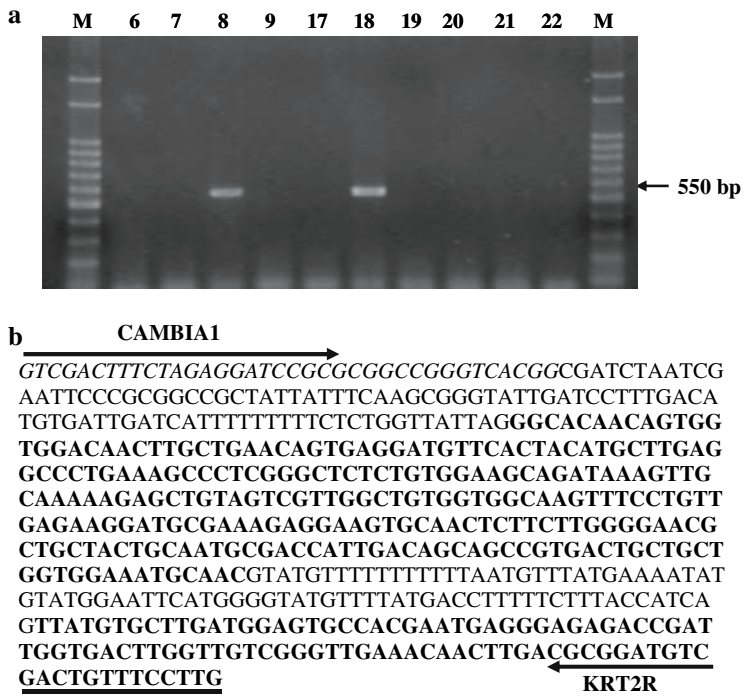

Fig. 5 Analysis of transposition events of the SA-induced transgenic rice harboring KCEH system. (**a**) PCR analysis of *KCEH* transposition with the primers CAMBIA1 and mKRT2R and the expected fragments. (**b**) Sequence of the empty donor site of the transposition events, leaving the sequences from CAMBIA vector (*italics*) and the truncated *epsps*, in which the exons are shown in *bold*. The primers CAMBIA1 and mKRT2R are indicated as *arrows*. M=100-bp marker

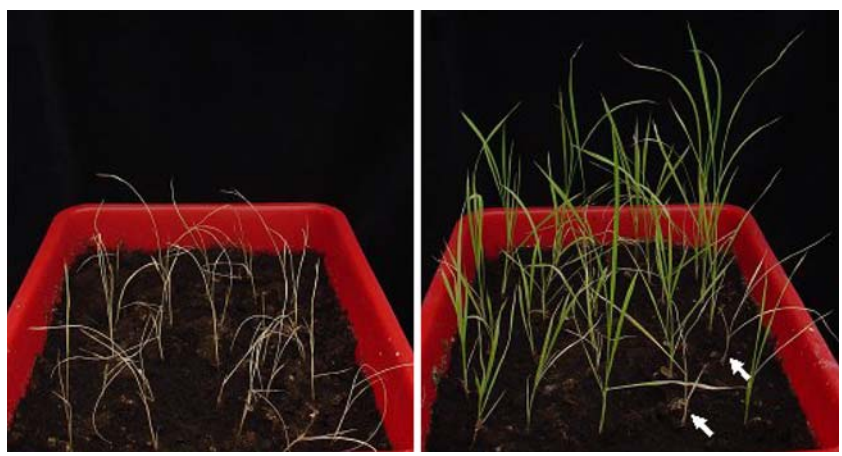

Fig. 6 Glyphosate-tolerance analysis of the self-pollinated progeny of *KCEH* transposed (*left*) and untransposed line (*right*). The *arrows* indicate the null KCEH progeny, which are not resistant to glyphosate

rice. We used glyphosate as the selection agent for rice transformation and found that the proliferation of the transgenic rice calli and subsequent shoot regeneration was as efficient as with hygromycin (Fig. 3). Another indication of a useful selectable marker gene is the copy number of the T-DNA integration sites. In transgenic rice, about 37% of transgenic rice contains one integrated T-DNA copy harboring *hpt* (Li et al. 2003), the most frequently used selectable marker gene in rice. Our results with glyphosate used as the selectable agent showed up to 43% of transgenic rice harboring a single transgene copy (Fig. 4). Furthermore, transgenic T1 progeny can be screened conveniently by applying commercial Roundup® (Fig. 6). All of these results suggested that *epsps* could be a suitable selectable marker.

With a transposon system to terminate the marker gene, the GOI-containing transposon could be used to create more independent transgenic lines from one successful transformant. We previously studied the behavior of another PR-1a::*TPase*-based inducible transposon in rice and found the transposons to be very active in SA-induced rice calli (Charng et al. 2007). We applied the same method to induce T1 rice

Table 1 Genomic sequences flanking *KECH* insertions in transgenic rice plants. The T-DNA integration site of each line is indicated after its designation

Line	Chromosome	BACs/PACs	Insertion position (bp)	GenBank accession no.	Identities
K-08 (21)	(T-DNA) 10	OSJNBb0060I05	56901	AC092697	86/86 (100%)
	(*KCEH*) 10	OSJNBa0040D23	32948	AC074196	102/102 (100%)
K-18 (12)	(T-DNA) 3	OSJNBa0039F10	52992	AC137931	127/128 (99%)
	(*KCEH*) 3	OSJNBa0045E22	156408	AC137072	217/217 (100%)
	(*KCEH*) 8	OSJNBb0092C08	74311	AP005391	251/251 (100%)
K-26	(T-DNA) 4	OSJNBb0022F23	130273	AL606447	227/227 (100%)
	nd	nd	nd	nd	nd
K-34	(T-DNA) 11	OSJNBa007P22	82765	AC109594	256/256 (100%)
	(*KCEH*) 11	OSJNBa0007P22	143401	AC109594	198/198 (100%)
K-40	(T-DNA) 5	OJ1362_D02	120545	AC105770	308/316 (97%)
	(*KCEH*) 5	OJ1281_H05	30721	AC117265	290/290 (100%)

nd=no detectable product obtained after TAIL PCR amplification

calli containing *KCEH* and found similar results for 16% somatic and 4.6% germinal transposition efficiency. Some transposon-containing transgenic lines have high transposition efficiency (Charng et al. 2007) and could be used as starters to create a sufficient number of marker-off transgenic lines. Although we have also observed that the *Ac*-based inducible transposons show a preference for linked transposition (Table 1 and Charng, unpublished results), the KCEH system was designed to truncate the marker gene, with no need for out-crossing.

One concern with the system is that tightly linked transposition can cause the marker gene to remain active. This possibility could be rare in plants. A linked transposition consists of four patterns: up- or down-stream integration plus forward or reverse of the marker's reading frame. Even if a tightly linked, downstream, forward transposition occurs, it can be ruled out by analysis of the flanking DNA sequences or glyphosate-tolerance activity. Indeed, Fig. 6 showed the loss of glyphosate-tolerance function in the progeny of a transposed transgenic rice line harboring the KCEH system.

Another application of the system of transposon-mediated break in the exons of a transgene is to terminate the expression of the transposase gene of *Ac* itself. Since the native *Ac* transposase gene contains four introns, we located one end of *Ac* in the intron of the transposase gene itself to yield an inducible self-stabilizing transposon, which has been useful for creating stable knockout mutants in rice (Charng et al., unpublished results). Therefore, the *Ac* 3′ end can be located in one intron of the native transposase gene, which would be replaced with the *TPase*, as shown in Fig. 1a. The induced transposition can truncate both *epsps* and *TPase* to terminate the marker gene and prevent further transposition. Alternatively, to accommodate a new marker gene into the KCEH system, one could implant another system to eliminate the complicated cloning procedure. Indeed, a Gateway-based system reported by Magnani et al. (2006) for multi-site cloning in one recombination event could imply more opportunities for application of the KCEH system.

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